DISCOVERY OF SELECTIVE ANTAGONISTS FOR THE α9α10 NICOTINIC ACETYLCHOLINE RECEPTOR

by

Sean Bradley Christensen

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The thesis of **Sean Bradley Christensen**

has been approved by the following supervisory committee members:

J. Michael McIntosh	, Chair	**9/9/2014** Date Approved
Grzegorz Bulaj	, Member	**9/9/2014** Date Approved
Gary J. Rose	, Member	**9/9/2014** Date Approved

and by **M. Denise Dearing** , Chair/Dean of

the Department/College/School of **Biology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are a class of ligand gated ion channels that are widely distributed in neuronal and non-neuronal cell types. One subtype of nAChRs, the α9α10 nAChR, is of particular interest as it has been implicated in pain signaling. Block of the α9α10 nAChR has demonstrated analgesia in several animal models. Because of the role of these receptors in pain, the discovery of antagonists of the α9α10 nAChR has important practical applications. *Conus* is a genus of venomous mollusks whose venom components have been widely utilized as pharmacological tools to discriminate between receptor and ion channel subtypes. Several species of *Conus* were selected and screened for activity against the α9α10 nAChR, with two of the venoms selected for further study. Subsequent purification led to the discovery of αS-GVIIIB, a novel σ-conotoxin that is potent for the α9α10 nAChR with an IC_{50} of 9.8 nM, and is over 100-fold more selective for the α9α10 nAChR compared to other nAChR subtypes. Furthermore, αS-GVIIIB gives increased insight into the σ-conotoxin family, a class of toxins that is not widely studied. Of particular interest is that the previously discovered σ-GVIIIA is selective for the 5-HT_3 serotonin receptor and the newly discovered αS-GVIIIB, from the same *Conus* species, targets a different class of ligand-gated ion channels. The understanding of σ-conotoxins was also furthered with the demonstration that αS-GVIIIB competes with α-RgIA for the ACh binding domain, illustrating that αS-GVIIIB is a competitive antagonist. Also, toxins from the αD-conotoxin family are potent for the α9α10 nAChR, but selectivity is not a key feature as the αD-conotoxins are also potent for several other nAChRs.

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CHAPTER 1

INTRODUCTION

Introduction

Background on Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptors (nAChRs) are a class of ligand gated ion channels that use acetylcholine (ACh) as their primary natural agonist. Each functional receptor is a pentamer containing five subunits; differing combinations of subunits determine the pharmacological role of the receptor [1]. There are seventeen known nAChR subunits; α2-α10 and β2-β4 are referred to as the neuronal subunits, and α1, β1, δ, ε, γ make up the muscle subtypes found at the neuromuscular junction. For the neuronal subtypes, typically a functional receptor is a heteromer comprised of both α and β subunits. There are also a few known functional homomers comprised of five subunits of α7 or α9 that form receptors, and α9α10 will form a receptor in the absence of a β subunit [2].

The α9α10 nAChR was originally identified in the cochlea as the receptor that mediates synaptic transmission between the olivocochlear efferents to auditory hair cells [3]. Subsequent studies have shown the presence of α9α10 nAChRs in non-neuronal tissues, including adrenal chromaffin cells, immune cells, and breast tumors [4] [5]. Several studies have demonstrated that block of α9α10 nAChRs is associated with analgesia [6] [7] [8]. Phylogenetically, the α9, α10, and α7 are considered the primordial receptor subunits, with the muscle subtypes coming next, and all the remaining neuronal subunits branching last [9]. Because of this relationship, the majority of antagonists that target α7, α9α10, or the muscle nAChR will have potency for all three subtypes. For example, a well-studied peptide α-bungarotoxin from the venom of the banded krait snake *Bungarus multicinctus* potently blocks all three receptor subtypes. While the understanding of the role of the α9α10 nAChR is increasing, unfortunately, there are few available ligands available to characterize the function and pharmacology of α9α10 nAChRs.

Conotoxins as Neuropharmacological Tools

The venom of predatory cone snails from the genus *Conus* have been extensively studied to yield new pharmacological tools that target a variety of ion channels with unique selectivity profiles [10]. There are approximately 500-700 species of *Conus* that use venom to prey upon worms, mollusks, and fish. Each individual species of venom is composed of roughly 100-200 peptide sequences that target different ion channels [11]. These toxin sequences have been grouped into families based upon peptide sequence similarities, cysteine framework, and the ion channel subtypes they target.

Conotoxin Superfamilies that Target Nicotinic Acetylcholine Receptors

Among the identified *Conus* toxin superfamilies, there are seven that have been shown to target nAChRs [12]. The most studied of these, the α-conotoxins, are competitive blockers of the ACh binding site and have shown potency for several of the neuronal subtypes and the muscle subtype of nAChRs [13]. The αA-conotoxins, also competitive for the ACh binding site, target mainly the muscle nAChR [14]. The αC-conotoxins and the ψ-conotoxins are two superfamilies that are noncompetitive blockers of the muscle nAChR [15] [16]. The αS-conotoxins target nAChRs and have a preference for the muscle subtype, but are potent blockers of neuronal nAChRs [17]. It was previously unknown whether the αS-conotoxins were competitive for the ACh binding site, but that was tested as part of this study. The αD-conotoxins also target several neuronal nAChR subtypes; they are noncompetitive blockers that are not selective among subtypes as they will block several with high potency [18]. Finally, the recently discovered αB3-conotoxin VxXXIVA is a unique toxin that weakly, with about a ~1 μM IC_{50}, targets the α9α10 nAChR [19].

Of the seven superfamilies of conotoxins that target nAChRs, previous work has shown

only two of those to target the α9α10 nAChR. Several of the well-studied α-conotoxins target the α9α10 nAChR as well as the aforementioned αB3-VxXXIVA. Previously published α-conotoxins that target the α9α10 nAChR are α-PeIA, α-RgIA, and α-Vc1.1 [20] [21] [7]. The first reported conotoxin antagonist of α9α10 nAChRs, α-PeIA, was useful in that it discriminated between α9α10 and α7, but unfortunately, it was not selective for α9α10 as it was potent for several other neuronal subtypes. The next two characterized peptides, α-RgIA and α-Vc1.1, were both selective for the α9α10 nAChR. But a drawback for both peptides is that they have rapid off-rates for the α9α10 nAChR. Also, while both are selective and potent for the α9α10 nAChR, this testing was done for the rat α9α10 nAChR; when tested on the human α9α10 nAChR, the potency is significantly reduced [22] [23]. These two peptides have been instrumental in the study of the properties of the α9α10 nAChR, but most of these studies have been in rodents and an ideal toxin would be potent for both human and rat α9α10 nAChR.

The scope of this project is to take a broad approach at discovering new antagonists of the α9α10 nAChR. The aforementioned previously identified antagonists were discovered by screening individual previously characterized compounds against the α9α10 nAChR. In an attempt to find additional antagonists using a broad approach, venoms from selected species of *Conus* were screened for activity against the α9α10 nAChR. With this method, roughly 100-200 compounds contained in each species venom can be screened together, and any venom that exhibits activity for the α9α10 nAChR can then be further studied to identify and characterize the active component(s) [24]. The advantage to this approach is that thousands of toxins can be screened in a short amount of time. The potential disadvantage is that the active component(s) could be hard to separate from the other venom components. Also, since the α9α10 nAChR is the target in this screening, there is also the potential that the active component(s) could have potent activity on other nAChR subtypes.

CHAPTER 2

VENOM SCREENING

Introduction

Seventeen venoms were initially selected for screening; these were selected from a variety of *Conus* clades representing all three major forms of predation (fish, mollusk, and worm) [25]. This would give a reasonable cross section of the genus *Conus*, and a wide variety of venom components should be present. The two desirable features are potency for the α9α10 nAChR, and a slow off-rate. Having a ligand with a slow off-rate would be a useful pharmacological tool that could be radiolabeled or tagged with a fluorescent dye to facilitate the visualization of the α9α10 nAChR in tissue preparations.

Methods

Preparation of Crude Venom Samples

Small aliquot samples of whole venom of each species of *Conus* were prepared as follows. For each individual species, 40 mg of lyophilized venom ducts were weighed into a test tube and 800 μL of B35 (65:35:0.1 H_2O/acetonitrile/ trifluoroacetic acid (TFA)) was added. Next, the venom duct samples were homogenized by hand using a single-use disposable pestle, with the sample being ground a minimum of thirty rotations. The samples were then centrifuged and the supernatant was removed from the pellet. The supernatant of each species was then split into aliquots representing 2 mg of the original venom sample and lyophilized for future screening purposes.

Oocyte Electrophysiology

Oocytes were micro-injected with an equal ratio of cRNA of the α9 and α10 nAChR subtypes and allowed to incubate for 1-3 days. Oocytes expressing α9α10 nAChRs were voltage clamped at -70 mV using a two-electrode voltage clamp system (Warner Instruments) as previously described [26]. Briefly, the oocytes were placed in a 30 μL bath and perfused with

ND96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.5) containing 0.1 mg/mL bovine serum albumin (BSA). Venom samples were applied to the bath and allowed to incubate for 5 minutes. Following incubation, ACh was applied at a concentration of 10 μM for 1 second followed by a 60 second wash, and the peak amplitude was recorded. For each species of *Conus* tested, initial screening started at 10 μg of crude venom, and if over 80% block was observed, the sample was diluted ten-fold and tested again.

Results

Various Venoms Screened

Whole venom preps were prepared as described and tested for activity against the α9α10 nAChR. Seventeen species were screened representing a broad array from the various *Conus* clades. Samples were initially screened at 10 μg of venom extract per application (Table 2.1). Any sample that elicited greater than 80% block was diluted ten-fold to 1 μg per application and tested again. For the 1 μg per application, five species remained that blocked greater than 80% of the ACh-induced current. Of the five remaining species, four were from the same *Conus* clade, designated rhizoconus (*C. vexillum*, *C. capitaneus*, *C. miles*, and *C. mustilinus*), and the other species was *C. geographus*. When diluted ten-fold more to 100 ng of crude venom per application, only *C. vexillum*, *C. capitaneus*, and *C. mustilinus* blocked over 80% of the ACh response. When tested at 10 ng, *C. capitaneus* still blocked 78% of the ACh response.

Discussion

From the venom screening against the α9α10 nAChR, several candidates appeared promising for further study. For the five species that blocked greater than 80% per 1 μg of venom application, all had a slow ACh response recovery after venom wash-out. Since four of the five species are closely related phylogenetically, they all belong to the rhizoconus clade, we

decided to select one species from this group for further investigation. Additionally, we decided to pursue the activity demonstrated by *C. geographus*, as this species is fairly diverse from the rhizoconus clade. The results from *C. geographus* are described in Chapter 3. From the rhizoconus clade, we selected *C. capitaneus* for further study because it was the most potent of the four species, and also because a peptide from *C. vexillum*, the aforementioned αB3-VxXXIVA, has a weak activity on the α9α10 nAChR [24]. The findings from *C. capitaneus* are presented in Chapter 4.

Table 2.1 Venom screening on α9α10 nAChRs

Species	% Block 10 μg	SEM	% Block 1 μg	SEM	% Block 100 ng	SEM	% Block 10 ng	SEM
C. vexillum	99.9	*	99.2	*	84.8	8.85		
C. capitaneus	99.7	*	99.8	*	94.9	*	77.5	*
C. mustelinus	99.8	*	99.8	*	99.4	*	56.9	*
C. miles	99.2	*	88.8	*	16.2	7.00		
C. rattus	92.4	*	13.4	4.58				
C. imperialis	86.9	*						
C. caracteristicus	99.0	*	52.6	7.95				
C. aulicus	46.7	1.44						
C. quercinus	53.7	7.37						
C. virgo	76.5	1.45						
C. litteratus	15.7	4.58						
C. geographus	98.8	0.38	84.7	3.42	5.2	2.75		
C. brettinghami	91.0	1.21	13.8	2.41				
C. radiatus	82.3	5.38	21.6	8.09				
C. consors	96.1	0.79	38.1	6.58				
C. ebraeus	50.2	*						
C. distans	29.7	*						

SEM, Standard Error of the Mean; *, less than 3 test applications.

CHAPTER 3

αS-CONOTOXIN GVIIIB POTENTLY AND SELECTIVELY BLOCKS α9α10 NICOTINIC ACETYLCHOLINE RECEPTORS

Sean B. Christensen, Pradip K. Bandyodpadhyay,

Baldomero M. Olivera, and J. Michael McIntosh

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αS-conotoxin GVIIIB potently and selectively blocks α9α10 nicotinic acetylcholine receptors

Sean B. Christensen [a], Pradip K. Bandyopadhyay [a], Baldomero M. Olivera [a], J. Michael McIntosh [a,b,c,*]

[a] Department of Biology, University of Utah, Salt Lake City, UT 84112, USA
[b] George E. Wahlen Veterans Affairs Medical Center, Salt Lake City, UT 84108, USA
[c] Department of Psychiatry, University of Utah, Salt Lake City, UT 84112, USA

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ABSTRACT

Although acetylcholine is widely utilized in vertebrate nervous systems, nicotinic acetylcholine receptors (nAChRs), including the α9α10 subtype, also are expressed in a wide variety of non-neuronal cells. These cell types include cochlear hair cells, adrenal chromaffin cells and immune cells. α9α10 nAChRs present in these cells may respectively play roles in protection from noise-induced hearing loss, response to stress and neuroprotection. Despite these critical functions, there are few available selective ligands to confirm mechanistic hypothesis regarding the role of α9α10 nAChRs. *Conus*, has been a rich source of ligands for receptors and ion channels. Here, we identified *Conus geographus* venom as a lead source for a novel α9α10 antagonist. The active component was isolated and the encoding gene cloned. The peptide signal sequence and cysteine arrangement had the signature of the σ-conotoxin superfamily. Previously isolated σ-conotoxin GVIIIA, also from *Conus geographus*, targets the 5-HT_3 receptor. In contrast, αS-GVIIIB blocked the α9α10 nAChR with an IC_{50} of 9.8 nM, yet was inactive at the 5-HT_3 receptor. Pharmacological characterization of αS-GVIIIB shows that it is over 100-fold selective for the α9α10 nAChR compared to other nAChR subtypes. Thus, the S-superfamily represents a novel conotoxin scaffold for flexibly targeting a variety of receptor subtypes. Functional competition studies utilized distinct off-rate kinetics of conotoxins to identify the α10/α9 nAChR interface as the site of αS-GVIIIB binding; this adds to the importance of the (+) face of the α10 rather than the (+) face of the α9 nAChR subunit as critical to binding of α9α10-targeted conotoxins.

1. Introduction

Conus are predatory marine mollusks that envenomate prey to facilitate capture. There are an estimated 500–700 species of cone snails. Each cone snail venom is comprised of a unique cocktail of hundreds of components. The *Conus* species therefore represent a natural, evolutionarily refined library of compounds that act on the nervous system [1–5].

Nicotinic acetylcholine receptors (nAChRs) are a subset of ligand gated ion channels that use acetylcholine (ACh) as its primary natural agonist [6]. To date, there are 17 known nAChR subunits in vertebrates, those found primarily in muscle that include α1, β1, δ, γ and ε and those found in neuronal as well as non-neuronal tissues, α2–α10, and β2–β4. These subunits combine to form pentamers with varying pharmacology and function that depends on the composition of the individual subunits. Additionally, α7, α9 and α10 can form functional receptors in the absence of β subunits; α7 and α9 form homomers, while α9α10 can form a functional heteromer. Phylogenetic data of nAChRs has shown that α7, α9 and α10 are closely related compared to the other neuronal subtypes and the muscle subtypes [7].

The α9α10 nAChR was originally identified as the receptor that mediates synaptic transmission from the olivocochlear efferents to auditory hair cells of the cochlea [8]. The α9α10 nAChR was subsequently identified in adrenal chromaffin cells and is upregulated in response to cold-induced stress [9]. Other studies

Abbreviations: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; HPLC, high performance liquid chromatography; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid..

* Corresponding author at: Department of Biology, The University of Utah, 257 S. 1400 E., Salt Lake City, UT, USA. Tel.: +801 581 8370; fax: +801 585 5010.
E-mail address: mcintosh.mike@gmail.com (J.M. McIntosh).

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suggest the presence of α9α10 nAChRs in tissues including immune cells and breast tumors [10,11]. Block of α9α10 nAChRs has been associated with analgesia [12–15]. Despite the potential importance of this receptor subtype, there are few available ligands with which to characterize the function and pharmacology of α9α10 nAChRs.

nAChRs are utilized by various prey types hunted by *Conus*. As such, nAChRs provide a rational target for prey immobilization. The α-conotoxins are a well-studied family of *Conus* peptides that have been shown to act on a variety of nAChR subtypes [16–20]. Recently, however, there have been reports of other families of conotoxins that have activity on nAChRs [21–25].

The aim of this study was to examine *Conus* venoms for the presence of uncharacterized antagonists of the α9α10 nAChR. To achieve this goal we screened several venom samples against the α9α10 nAChR. We then purified and characterized the responsible component from the most potent venom. The novel peptide αS-GVIIIB was identified and characterized.

2. Materials and methods

2.1. Crude venom extraction

Various species of *Conus* were selected from several clades [26]. To 40 mg of each venom was added 800 μl of B35 (65:35:0.1 H_2O/acetonitrile/trifluoroacetic acid) (Thermo Fisher Scientific). The mixtures were homogenized by hand using a disposable pestle a minimum of 30 rotations or until the tissue appeared to be thoroughly dissociated. The samples were then centrifuged at 13,000 RPM and the supernatant was removed. The venom was then re-extracted a second time and the supernatants from both extractions were pooled for each individual species.

For large scale extraction of *Conus geographus*, 200 mg of lyophilized venom was combined with 2 ml of B35. The sample was homogenized using a Teflon pestle spinning at 1000 RPM. The sample was then centrifuged at 13,000 RPM and the supernatant was removed. An additional 2 ml of B35 was added to the pellet and homogenization and centrifugation were repeated two additional times. The combined supernatants were then diluted to a volume of 50 ml using Buffer A (99.9:0.1 H_2O/trifluoroacetic acid) and loaded onto a semi-preparative C18 HPLC column (Vydac 218TP510, Grace Discovery Sci.). The gradient was 2% to 100% B60 (40:60:0.092 H_2O/Acetonitrile/trifluoroacetic acid) for 98 min (1% per min). The flow rate was 5 ml/min; fractions were collected for the length of the run at 2 min intervals.

2.2. Venom purification and isolation

The resulting fractions from the large scale venom extraction described above were assessed for activity on α9α10 nAChRs. Fractions exhibiting activity were selected for further purification to isolate the active component. The active fraction was run on an analytical HPLC (Alliance, Waters Corp.) using a Vydac C18 column (218TP54, Grace Discovery Sci.) and a gradient of 10–50% B60 for 40 min (1% per min). The flow rate was 1 ml/min. All peaks were collected and assessed for activity on oocytes expressing α9α10 nAChRs. Active peaks were then further purified using size exclusion chromatography (SEC) as follows: two columns (Superdex Peptide HR 10/30, GE Healthcare Bio-Sciences Inc.) were attached in series and eluted with B30 (70:30:0.1 H_2O/acetonitrile/trifluoroacetic acid) at a flow rate of 0.5 ml/min.

2.3. Peptide sequencing

The isolated active peptide was reduced using dithiolthreitol (DTT) (Promega, Madison, WI) at a concentration of 20 μM. To 5 nmol of lyophilized peptide was added 25 μl of 1 M Tris (pH 7.5) (Sigma–Aldrich), 2.5 μl of 0.1 M EDTA (pH 7.5) (Sigma–Aldrich), and 1.6 mg DTT dissolved in 223 μl H_2O. The reaction proceeded for 2 h at room temperature, and was quenched with 8% formic acid (Sigma–Aldrich) (20 μl for the 250 μl reaction volume). Following DTT reduction, the Cys residues were alkylated with 4-vinylpiridine (Sigma–Aldrich) [27]. 250 μl of Buffer A was added to the reaction mix and 3 μl of 4-vinylpiridine was added. Immediately after addition of the 4-vinylpiridine the reaction mixture was bubbled with Argon and then was incubated at room temperature for 30 min in the dark. The peptide was purified using an analytical HPLC. MALDI-TOF mass spectrometry was performed to confirm the reduction of disulfide bonds and pyridylethylation of the Cys residues. Pyridylethylated peptide was sequenced using Edman chemistry on an Applied Biosystems 477A Protein Sequencer at the Protein/DNA Core Facility at the University of Utah.

2.4. Peptide quantification

The concentration of purified αS-GVIIIB was quantified using an absorbance measurement with a spectrophotometer at a wavelength of 280 nm and calculated using the Lambert–Beer equation, $A = \varepsilon lc$; where A is absorbance, ε is the extinction coefficient, l is the cuvette path length and c is the concentration. The cuvette path length is 1 cm and the extinction coefficient for αS-GVIIIB at a wavelength of 280 nm was calculated as 2480 $cm^{-1}M^{-1}$ using a peptide properties calculator found at www.basic.northwestern.edu/biotools/proteincalc.html.

2.5. cDNA cloning

We used the sequence from a previously published *Conus geographus* transcriptome [28] to design two forward primers for carrying out nested polymerase chain reactions, designated Oplus: 5′GCAAGACGTGACGTGCAAG 3′ and Iplus: 5′CATGATGTCGAAAATGGGAGC 3′. First strand cDNA was synthesized from total RNA isolated from venom duct using 3′-RACE CDS primer A (SMARTer™ RACE cDNA Amplification Kit, Clontech Laboratories, Inc.) according to the vendor's instructions. cDNA encoding the conotoxin was isolated by amplification using polymerase chain reaction (PCR). The initial PCR was carried out using Advantage 2 polymerase (Clontech) and oligonucleotides, Oplus and UPM (Clontech, kit mentioned above) as primers. The amplified product was diluted 50 fold and used as template for a subsequent PCR using Go Taq® DNA polymerase (Promega Corporation, Wi) and Iplus and NUP(kit mentioned above) as primers (PCR was carried out using buffers and instructions provided by the vendors). The amplified product was purified from an agarose gel using QIAquick Gel Extraction Kit (Qiagen Inc., CA). The isolated DNA was ligated to pGEM®-T Easy vector DNA (Promega, Wi) and used to transform *E. coli* DH10B (New England Biolabs Inc.). Vector DNA carrying the insert was isolated and their sequences determined by Sanger's dideoxy sequencing method at the University of Utah DNA Peptide Core facility.

2.6. Oocyte electrophysiology

Xenopus laevis (*Xenopus* express, FL) oocytes were microinjected with cRNA of the various rat nAChR subunits as previously described [29]. Clones for α9 and α10 were generously provided by AB Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina), high expressing β2 and β3 were generously provided by C Luetje (University of Miami, Miami, FL) and all other nAChR subunits used were generously provided by S Heinemann (Salk Institute, LaJolla, CA). The 5-HT_3 serotonin receptor was generously

provided by AV Maricq (University of Utah, Salt Lake City, UT) [30]. Injected oocytes were incubated at 17 °C for 1–3 days. Following that, oocytes expressing the various receptors were voltage clamped at −70 mV using a two-electrode voltage clamp system (Warner Instruments, Hamden, CT) as previously described [29]. Briefly, oocytes were placed in a 30 μl bath and perfused with ND96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.5) (Thermo Fisher Scientific) containing 0.1 mg/ml protease-free bovine serum albumin (BSA) (Sigma–Aldrich). ACh (Sigma–Aldrich) was applied for 1 s at 1 min intervals and the peak amplitude was recorded. ACh concentration was 10 μM for α9α10 nAChR and human adult muscle; 300 μM for α7 nAChR; and for all other nicotinic receptors ACh concentration was 100 μM. For the oocytes expressing the 5-HT_3 receptor a 1 s pulse of 5 μM serotonin (Sigma–Aldrich) was applied at 1 min intervals [31]. After a stable baseline of agonist-responses was achieved, the solution flow was stopped and whole venom or venom fraction was applied and allowed to incubate for 5 min. Following incubation, agonist was pulsed and response was measured. For purified peptides, αS-GVIIIB at concentrations of 30 nM or lower, or αS-RVIIIA at concentrations of 100 nM or lower, a solution of ND96 containing the toxin was perfused onto the oocyte at a flow rate of ~1 ml/min until steady state block was achieved. Response was monitored by applying a 1 s agonist pulse once per minute. For higher toxin concentrations, the flow was stopped and toxin was applied and allowed to incubate for 5 min before flow of buffer was resumed.

3. Results

3.1. Activity of conus crude venoms

Conus venoms are composed of hundreds of individual compounds. Each *Conus* species has its own unique set of peptides, and thus there are tens of thousands of unique peptides present in these venoms. The purification and testing of each of these individual components is impractical. We therefore prepared whole venom extracts from representative species from eight various clades of *Conus* [26] as described in *Experimental Procedures* and tested these for activity against the α9α10 nAChR heterologously expressed in *Xenopus* oocytes. Venom samples were initially screened using 0.33 μg/ml of venom extract per test application. Any sample that elicited greater than 80% block of the α9α10 nAChR was subsequently diluted 10-fold and tested again at a concentration of 0.033 mg/ml. Venom from a single species, *Conus geographus*, blocked greater than fifty percent of the ACh induced current when tested at 0.033 mg/ml (~84% block) (Table 1).

3.2. Purification of conus geographus venom

From the initial screening on the α9α10 nAChR, *Conus geographus* was selected based on its potent block of the receptor and also because the block was slowly reversible following venom washout, suggesting slow off-rate kinetics and possibly high potency of the responsible compound(s). A larger scale extraction of venom (200 mg) was performed and separation of the venom components was done based on hydrophobicity using reversed-phase HPLC (Fig. 1A). Ten ml fractions were collected and lyophilized. In the original assay we had tested 0.033 mg/ml of crude venom extract, a control sample was taken from the 200 mg extraction before fractionation and it exhibited 96.3% block for 0.033 mg/ml. We defined the activity present in 1 μg of venom as one 'unit'. From a 200 mg crude venom preparation we assumed that we would have 200,000 units of material. Therefore, 100 μl of each fraction was lyophilized and re-suspended in 60 μl of ND96 buffer and then serially diluted such that one part in 200,000 of each original fraction was tested for activity on oocytes expressing α9α10 nAChRs. The fraction eluting at 32–34 min blocked 92.4% of the ACh response (Fig. 1A).

A second round of purification of the identified active fraction was performed based on hydrophobicity using a reversed-phase C18 column (Fig. 1B). Forty nmol of toxin was obtained from the original starting material of 200 mg of crude venom. α9α10 nAChR expressing oocytes were again used to screen activity and the responsible component was identified (Fig. 1B). The active material was further purified based on molecular mass using a size-exclusion column (Fig. 1C). The resulting fractions were tested on

Table 1
Crude *Conus* venoms activity on α9α10 nAChRs.

species	10 μg % block	SEM	1 μg % block	SEM
C. aulicus	46.7	1.4	ND	
C. quercinus	53.7	7.4	ND	
C. virgo	76.5	1.5	ND	
C. litteratus	15.7	4.6	ND	
C. geographus	98.8	0.4	84.7	3.4
C. brettinghami	91.0	1.2	13.8	2.4
C. radiatus	82.3	5.4	21.6	8.1
C. consors	96.1	0.8	38.1	6.6

SEM, standard error of the mean; ND, not determined.

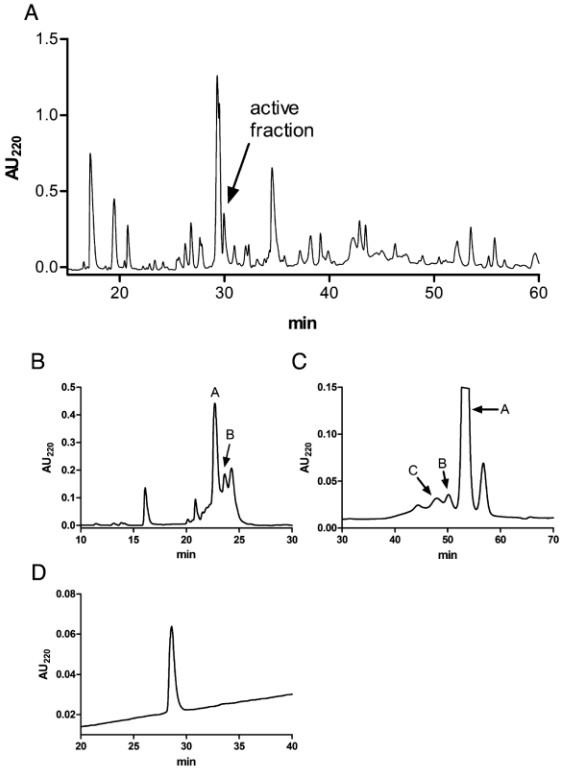

Fig. 1. HPLC purification of αS-GVIIIB. (A) Purification of crude venom extract of 200 mg of lyophilized *Conus geographus* venom. Arrow indicates the fraction that blocked α9α10 nAChRs. B. Analytical scale purification of 100 μl of the active fraction from panel A; Peaks A and B were active. (C) Size-exclusion chromatography of the active components shown in panel B. Peak C was the active component. (D) RP-HPLC of purified αS-GVIIIB (peak C).

α9α10 nAChRs. Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry showed a monoisotopic mass of 4435.6 Da (C) for the active component (Fig. 1D), and the two inactive side components shown in Fig. 1C had respective masses of 1417.2 Da (A) and 3768.8 Da (B). The active component purity was > 98% based on the UV absorption peaks obtained at 220 nm during HPLC (Fig. 1D). Mass spectrometry of the active fraction showed a predominant main mass of 4435.6 Da; In addition, there were minor masses of 4419.6 and 4451.6 Da, which are −16 and +16 Da. These likely correspond to variable hydroxylation of Pro residues as has been reported for other conotoxins [2].

3.3. Sequencing of the active component

Previously characterized conotoxins generally have multiple Cys residues connected by disulfide bonds. We assumed that this was likely the case with the present compound, and we therefore reduced the compound with DTT and alkylated the active component with 4-vinylpyridine. The resulting product was purified by HPLC and analyzed by mass spectrometry; pyridylethylation resulted in a compound with an increased mass of 1054.2 Da, indicating the presence of 10 cysteine residues. The alkylated peptide was then subjected to Edman degradation sequencing that resulted in only a partial amino acid sequence (Fig. 2A). Using the partial amino acid sequence obtained, search results of previously published *Conus geographus* transcriptomes revealed two precursor proteins that contained a mature toxin sequence identical to that of the partial peptide sequence [28,32]. The two predicted mature toxins from the transcriptomes differed by only a single amino acid in the mature toxin region, a Pro/Arg substitution at the 35th amino acid. One of these sequences (the Pro variant) was identical to that of the sequence of the peptide isolated in the present work. To confirm the presence of this sequence in our collection of *Conus geographus*, a cDNA library was prepared from venom duct, and cloning was performed using primers designed from the transcriptome sequence as described in the *Experimental Procedures*. The cDNA sequencing results yielded clones with a Pro in the 35th position of the toxin sequence (Fig. 2B). The calculated mass of the two transcriptome

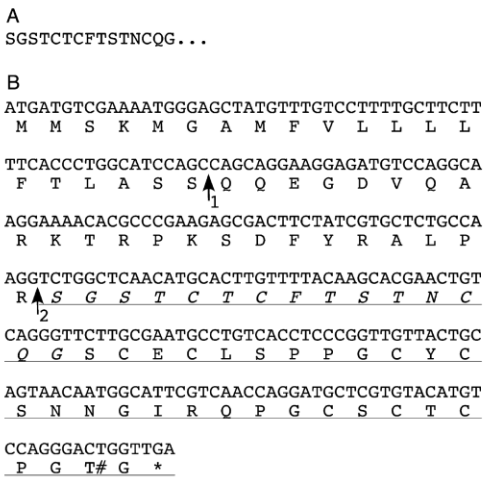

Fig. 2. Sequence of αS-GVIIIB. (A) Partial toxin sequence obtained from Edman degredation sequencing. (B) Gene sequence for αS-GVIIIB obtained from cDNA cloning. The first arrow represents the end of the signal sequence and start of the pro-peptide region. The second arrow represents the proteolytic processing site, and the mature toxin sequence is underlined. The C-terminus is predicted to be amidated, represented by #. The * represents the stop codon.

sequences are 4462.7 Da for the Arg variant and 4403.7 Da for the Pro variant. The measured mass of the venom isolated peptide was 4435.6 Da, which is +32 Da compared to that of the Pro variant. The increase of 32 Da is most likely attributable to hydroxylation of two of the four Pro residues in the peptide sequence. Hydroxylation of Pro is a common post-translational modification of *Conus* peptides [2]. The sequence of the pre-pro-region of the peptide places this peptide in the S-superfamily [33]. The Cys framework is that of conotoxins designated with Roman numeral VIII [34]. The peptide was therefore named αS-GVIIIB according to previously established criteria [35].

3.4. nAChR subtype selectivity testing

Several concentrations of αS-GVIIIB were applied to oocytes expressing α9α10 nAChRs. A concentration–response curve was generated yielding an IC_{50} of 9.8 nM (95% confidence interval of 6.6–14.4 nM) (Fig. 3A). Additionally, αS-GVIIIB was tested on several other neuronal nAChR subtypes, the adult muscle subtype α1β1δε, and also the 5-HT_3 serotonin receptor (Figs. 3B, C, and 4). Application of 100 nM αS-GVIIIB resulted in little or no block: % response was 96.3 (2.9)%, α3β2; 89.22 (7.7)%, α6/α3β2β3; 101.34 (6.5)%, α3β4; 95.40 (5.3)%, α4β2; and 97.22 (4.1)% for α7; (=S.E.M.). At 10-fold higher concentration partial block was observed, with the α3β2 and α6/α3β2β3 exhibiting 48.3% and 54.0% response to ACh, respectively (Fig. 4; Table 2). The small amount of available material prevented more detailed testing at higher concentrations.

3.5. Binding site analysis

We next examined the site of action of αS-GVIIIB. We specifically tested whether the binding of αS-GVIIIB is prevented by pre-incubation with the competitive α9α10 antagonist α-conotoxin RgIA [36,37]. Although α-RgIA potently blocks the α9α10 nAChR, it has relatively rapid off-rate kinetics. Ten μM α-RgIA completly blocked α9α10 nAChRs with full recovery 1–2 min after toxin washout (Fig. 5A) [36]. In contrast block by αS-GVIIIB is much more slowly reversed. One hundred nM αS-GVIIIB blocked ~90% of the ACh response with a slow recovery after toxin washout, half time to recovery is 7.1 min (n=3, 95% confidence interval, 6.2–8.4 min). We took advantage of this difference in off-rate kinetics to examine whether αS-GVIIIB and α-RgIA bind to the same or overlapping sites on the receptor. Using oocytes expressing α9α10 nAChRs, a baseline ACh response was established; then 10 μM α-RgIA was applied and allowed to incubate for 1 min. After pre-incubation with α-RgIA, 100 nM αS-GVIIIB was also added to the oocyte chamber and incubation in the presence of both toxins continued for another 4 min. Following the incubation, ND96 perfusion resumed and ACh pulses were taken at 1 min intervals (Fig. 5B). After application of both toxins, the ACh response was completely blocked. However, after 1 min of toxin washout, the ACh response returned to pre-toxin baseline levels. Thus, washout kinetics of toxin block were the same as that of α-RgIA but not that of αS-GVIIIB. Pre-application of α-RgIA prevented the slowly reversible block of αS-GVIIIB consistent with pre-binding of α-RgIA preventing subsequent binding of αS-GVIIIB.

We then conducted the converse experiment; that is pre-binding of αS-GVIIIB followed by subsequent exposure to α-RgIA. 100 nM αS-GVIIIB was first applied to the oocyte and allowed to incubate for 4 min; then, 10 μM α-RgIA was applied and the incubation in the presence of both toxins continued for 1 min (Fig. 5C). Under these conditions, the α9α10 nAChR was fully blocked. Toxins were then washed out and further ACh pulses showed slow recovery from toxin block back to baseline levels (Fig. 5C). Thus, the order of toxin exposure determined the

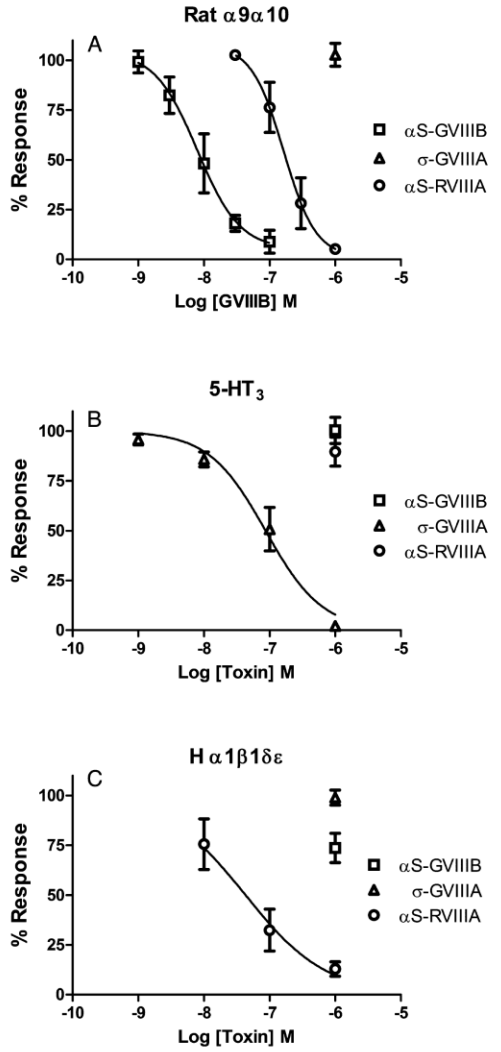

Fig. 3. Concentration–response of native sigma-conotoxins. αS-GVIIIB as well as two previously isolated sigma conotoxins were each tested on (A) α9α10 nAChRs, (B) the 5-HT_3 serotonin receptor, and (C) the muscle subtype (α1β1δε) nAChR, expressed in *Xenopus laevis* oocytes. αS-GVIIIB blocked the α9α10 nAChR with an IC_{50} of 9.8 (6.6–14.3) nM and a Hill slope of 1.32 (0.7–1.9); for 5-HT_3 and α1β1δε nAChR 1 μM toxin blocked less than 50% of the response. σ-GVIIIA blocked the 5-HT_3 receptor with an IC_{50} of 86.8 (56–135) nM and a Hill slope of 1.00 (0.6–1.4); for α9α10 and α1β1δε nAChRs, 1 μM toxin blocked less than half of the response. αS-RVIIIA blocked the α9α10 nAChR with an IC_{50} of 187 (138–255) nM and a Hill slope of 1.9 (1.0–2.9), α1β1δε with an IC_{50} of 43.1 (17–108) nM and a Hill slope of 0.71 (0.3–1.1); 1 μM toxin blocked less than 50% of the serotonin-evoked response of the 5-HT_3 receptor.

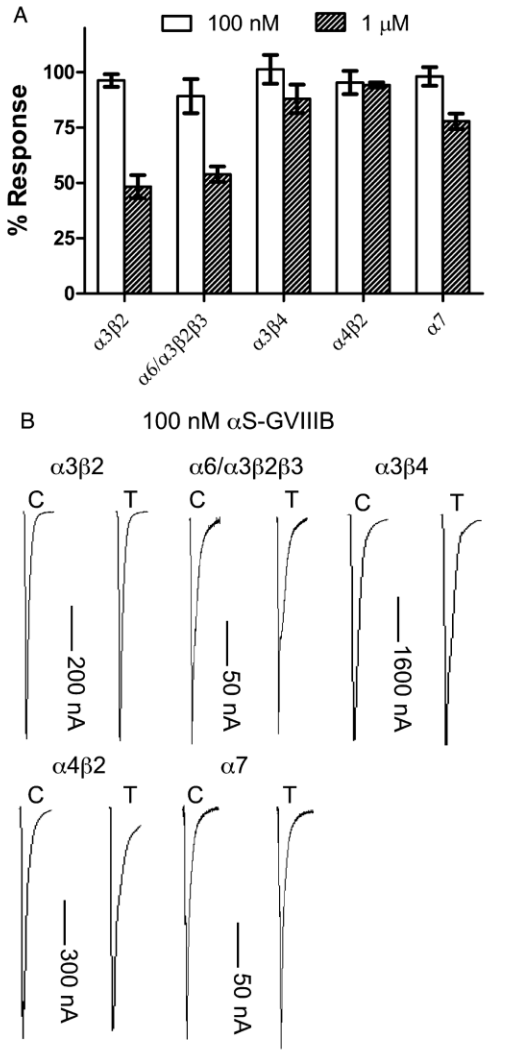

Fig. 4. Activity of αS-GVIIIB on other neuronal nAChRs. Oocytes expressing different nAChR subtypes were exposed to 100 nM and 1 μM applications of αS-GVIIIB for 5 min; after toxin incubation, a 1 s pulse of ACh was applied. (A) Each bar represents the average percentage of ACh response for the corresponding receptor subtype and αS-GVIIIB concentration ($n = 3$–5 oocytes per toxin concentration). (B) A representative trace for each receptor subtype exposed to 100 nM αS-GVIIIB. For each subtype, the first response, labeled C, is the control ACh response. The oocyte was then incubated for 5 min with 100 nM αS-GVIIIB, followed by another 1 s pulse of ACh, labeled T.

recovery from toxin block. When α-RgIA was applied first, the off-rate was similar to that with block by α-RgIA alone. In contrast, when αS-GVIIIB was applied first, the off-rate was similar to that with block by αS-GVIIIB alone. These results indicate that pre-binding of α-RgIA prevents subsequent binding of αS-GVIIIB. These findings are consistent with the toxins binding to the same, or overlapping binding sites. Block by 30 nM αS-GVIIIB (% response = 18.11 ± 4.0 S.E.M.) could be fully overcome by co-application of 300 μM ACh and 30 nM αS-GVIIIB (% response 99.47 ± 0.9 S.E.M.), also consistent with competitive binding.

4. Discussion

In this study we describe the purification and characterization of a novel conotoxin from *Conus geographus*, designated αS-GVIIIB. *Conus geographus* venom was chosen from the tested venoms based on the high degree of block and slow washout kinetics from application of a small amount of venom. The active peptide was

Table 2
αS-GVIIIB selectivity.

nAChR subtype	IC_{50}
α3β2	~1 μM
α6/α3β2β3	~1 μM
α3β4	>1 μM
α4β2	>1 μM
α7	>1 μM
α9α10	9.79 (6.6–14.4) nM
α1β1δε	>1 μM
5-HT_3	>1 μM

(), 95% confidence interval.

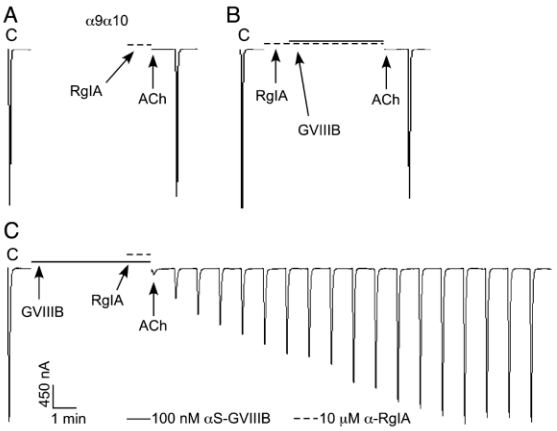

Fig. 5. Competition of α-RgIA and αS-GVIIIB for binding the α9α10 nAChR. (A) Washout kinetics of toxin block by 10 μM α-RgIA. A baseline control response was established for α9α10 nAChRs; the ND96 flow was then stopped for 5 min, and α-RgIA was added for the last minute. One s ACh pulses were then given at 1 min intervals. Note the rapid recovery from toxin block. (B) Washout kinetics of toxin block following pre-incuabation with RgIA. Baseline control responses to ACh were established and then ND96 flow was stopped. Ten μM α-RgIA was then applied to the bath, with a 1 min incubation. Subsequently, 100 nM αS-GVIIIB was added for an additional 4 min. ACh was pulsed at 1 min intervals. Note the rapid recovery from toxin block. (C) Washout kinetics of toxin block following pre-incubation with αS-GVIIIB. Oocytes were incubated with 100 nM αS-GVIIIB for 4 min. Ten μM α-RgIA was added to the bath for the final minute; then ACh pulses were resumed and given at 1 min intervals. Note the slow recovery from toxin block; note that washout kinetics are dependent on order of addition of toxins. Traces shown are representative, all experiments were repeated for a $n = 3$.

isolated using activity at the α9α10 nAChR as an assay to purify the responsible component. αS-GVIIIB is a 4 kDa, 10 Cys peptide that blocked the α9α10 nAChR with low nanomolar potency. By comparison, a well-known peptide, α-bungarotoxin from the snake venom of *Bungarus multicinctus*, also potently blocks the α9α10 nAChR. However, the larger (~8 kDa) α-bungarotoxin also potently blocks the α7 nAChR as well as the muscle nAChR subtype. In contrast, αS-GVIIIB is over 100-fold selective for α9α10 nAChRs compared with other nAChR subtypes (Fig. 4). α9, α10 and/or α7 subunits may represent primordial subunits of the nAChR subunit family [38]. As such, α9, α10 and/or α7 are more closely related and may explain why snake venom toxins and plant alkaloids have difficulty pharmacologically distinguishing among nAChRs containing these subunits.

Other previously characterized conotoxins also block the α9α10 nAChR. These peptides come from the worm-hunting *Conus regius* and *Conus vexillum*, and the mollusk-hunting *Conus victorea*. In contrast, αS-GVIIIB comes from a fish-hunting cone snail. The sequence and Cys arrangement of αS-GVIIIB are entirely unrelated to the α9α10 nAChR blocking peptides from the aforementioned species (Table 3). This demonstrates an example of convergent evolution, as the snail venom components diversify to different gene superfamilies with activity for the same receptor.

Recent work on conotoxins has indentifed whether venom components are used for prey capture or for defensive strategies. The study performed by Dutertre et al. [32] focused on the classification of *Conus geographus* venom. The previously characterized σ-GVIIIA was identified among the defensive venom components, as were several α-conotoxins that target nAChRs. Due to these reported findings, αS-GVIIIB may also be one of the toxins utilized by *C. geographus* to defend against predators.

The precursor sequence and arrangement of Cys residues place αS-GVIIIB in the sigma conotoxin family (Table 4). The selectivities of the three characterized σ-conotoxins are completely different. Whereas αS-GVIIIB selectively targets the α9α10 nAChR, σ-GVIIIA targets the 5-HT_3 receptor and αS-RVIIIA blocks multiple nAChR subtypes. It is of note that αS-GVIIIB comes from the same species as σ-GVIIIA and is from the same gene superfamily, yet the two toxins target two different classes of ligand-gated ion channels. Typically, the venom of cone snails will contain multiple toxins from the same gene family, but these toxins target the same receptor or ion channel class. The previous study on σ-GVIIIA tested activity on several nAChR subtype combinations, but did not test the α9α10 nAChR [39]. We therefore tested σ-GVIIIA on α9α10 nAChRs, and observed no block at a concentration of 1 μM (Fig. 3A). When 1 μM of αS-GVIIIB was applied to oocytes expressing the 5-HT_3 receptor, no block was observed (Fig. 3B). These results illustrate the stark preference for one type of ligand-gated ion channel between the two toxins from the same gene family, produced by the same *Conus* species. αS-RVIIIA, is a sigma family conotoxin from the fish-hunting *Conus radiatus*. Concentration response analysis indicated that αS-RVIIIA most potently blocked the muscle nAChR with an IC_{50} of 43 nM (Fig. 3C). αS-RVIIIA also blocked the α9α10 nAChR with an IC_{50} of 187 nM (Fig. 3A).

Neuronal nAChRs typically consist of both an α and a β subunit. A ligand binding interface occurs between these two subunits, with agonist binding that occurs between the (+) face of the α subunit and the (−) face of the β subunit. Binding of agonist causes closure of a loop of amino acids present in the α subunit (the C-loop) of the nAChR; movement of this loop is believed to facilitate subsequent opening of the ion channel. For these and other reasons, the α subunit is often referred to as the 'principal' binding subunit, whereas the β subunit is referred to as the 'complementary' binding subunit. The α9α10 nAChR is unique in that it is formed by two different α subunits and thus an asymmetrical binding interface is formed between two α subunits. However, if injected

Table 3
α9α10 nAChR-targeting conotoxins.

Conotoxin	Superfamily	Sequence	IC_{50}	Ref.
α-RgIA	A	GCCSDPRCRYRCR	4.6 nM	[36]
α-Vc1.1	A	GCCSDPRCNYDHPEIC-amide	109 nM	[43]
αB-VxXXIVA	B3	VRCLEKSGAQPNKLFRPPCCQKGPSFARHSRCVYYTQSRE	1.2 μM	[51]
αS-GVIIIB	S	SGSTCTCFTSTNCQGSCECLSPPGCYCSNNGIRQRGCSCTCPGT-amide	9.8 nM	This study

Table 4
Sequence alignment of venom purified σ-conotoxins.

Toxin	Signal sequence	Propeptide
αS-GVIIIB	MMSKMGAMFVLLLLFTLASS	QQEGDVQARKTRPKSDFYRALPR
σ-GVIIIA	MMSKMGAMFVLLLLFTLASS	LQEGDVQARKTRLKSDFYRALARDDR
αS-RVIIIA	MMSKMGAMFVLLLLFTLASS	QQEGDVQARKTHPKREFQRILLRSGR
	Mature toxin	
αS-GVIIIB	SGST**C**T---**C**FTSTN--**C**QGS**C**E**C**LSPPG**C**Y**C**SNNGIRQR-G**C**S**C**T**C**PGT	
σ-GVIIIA	---G**C**TRT-**C**GGPK---**C**TGT**C**T**C**TNSSK**C**G**C**RYNVHPSGWG**C**G**C**A**C**S	
αS-RVIIIA	---K**C**NFDK**C**KGTGVYN**C**GES**C**S**C**EGLHS**C**R**C**TYNIGSMKSG**C**A**C**I**C**TYY	

The toxin sequences are aligned based on the cys residues. The cys (C) are bold.

alone, the α9 subunit will form a functional homopentamer [40]. In contrast, the α10 subunit does not form a functional receptor when injected in the absence of other nAChR subunits [41]. For this reason, it was previously assumed that the α9 subunit was analogous to other nAChR α subunits in providing the principal binding site, whereas the α10 subunit functions similar to neuronal nAChR β subunits in providing a complementary binding site. However, α-conotoxins that bind to the α9α10 nAChR appear to bind to an interface formed by the α10/α9 subunit interface rather than the α9/α10 interface [37,42,43]. We therefore sought to examine whether block of the α9α10 nAChR by αS-GVIIIB occurs at the same or different binding site than the α-conotoxins. Block by α-RgIA reverses quickly after toxin washout. In contrast, block by αS-GVIIIB is more slowly reversed. We took advantage of the difference in off-rate kinetics to assess whether αS-GVIIIB and α-RgIA have similar binding sites. Pre-incubation of α-RgIA prevented subsequent block by αS-GVIIIB suggesting that the two peptides have the same or overlapping binding sites. Thus, an entirely different antagonist, αS-GVIIIB, also appears to bind to the α10/α9 rather than the α9/α10 subunit interface. Thus, at least for the tested conotoxins, the 'principal' face of the α9α10 nAChR appears to be provided by the α10 rather than the α9 subunit [44–46].

The 5-HT_3 receptor and nAChRs are members of the Cys-loop family of ligand-gated ion channels [47,48]. Both members of this family are composed of five symmetrically arranged subunits that surround an ion-conducting pore. In both cases, the extracellular domain is the site of action of agonists and competitive antagonists. The binding site for agonist and antagonists lies between the faces of two adjacent subunits and is formed by the convergence of three 'loops' (A–C) from the 'principal' subunit and three 'loops' (D–F) from the adjacent or 'complementary' subunit. These similarities likely allow the σ-conotoxins to be able to target one or another of these receptor types. However, significant differences are present with respect to key binding determinants of the 5-HT_3 receptor compared to the α10 and α9 subunits (Fig. 6) [44,49]. Although the binding determinants of αS-GVIIIB have yet to be determined, similar differences may account for specificity differences among sigma conotoxins.

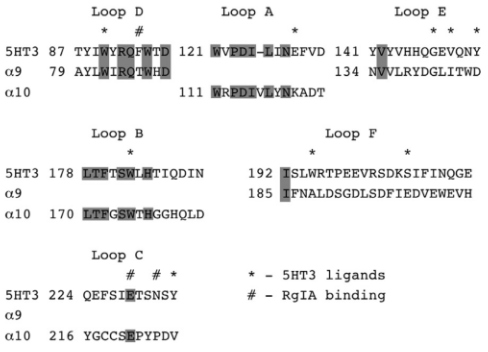

Fig. 6. Extracellular binding domain comparison of the 5-HT_3 receptor and α9α10 nAChR. The binding loops of the 5-HT_3 receptor were aligned with the corresponding loops of the α9 and α10 nAChR subunits. For the α9 nAChR subunit, loops D, E and F from the 'complimentary face' are indicated; for the α10 nAChR subunit the A, B and C loops from the 'principal face' are shown. * = residues shown to be important for agonists and competitive antagonists of the 5-HT_3 receptor. # = residues shown to be important for the α9α10 selective antagonist α-conotoxin RgIA. Conserved residues are shaded.

In conclusion, we have isolated and characterized a novel peptide from a fish-hunting cone snail. Although this peptide belongs to the σ-conotoxin superfamily, the selective block of the α9α10 nAChR makes it unique among previously characterized σ-conotoxins. Other cloning studies have identified several other σ-conotoxin sequences [33,50]; the pharmacological activity of these other toxins is unknown. The principal challenge to the synthesis of σ-conotoxins is the presence of 10 Cys residues. For ten cysteine residues, there are 945 unique possible ways to arrange the disulfide bonds. To-date, the disulfide arrangement has not been solved for any conotoxin superfamily containing more than eight cysteine residues. σ-conotoxins have now been discovered in cone snails that hunt a wide diversity of prey type (fish, mollusk and worm). The diversity of *Conus* species that use σ-conotoxins and the array of activity at various receptors, suggest that the σ-conotoxin superfamily has substantial potential to provide many more novel ligands for probing the structure and function of a variety of ligand-gated ion channel subtypes.

Acknowledgements

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CHAPTER 4

CONUS CAPITANEUS

Introduction

As mentioned in Chapter 2, the venoms screened from the rhizoconus clade all showed potent activity on the α9α10 nAChR. *Conus capitaneus* was selected for further workup, because its venom was the most potent of those in the clade, and showed long off-rate kinetics block. The start of the process was similar to that done for *C. geographus*, but sequence identification and selectivity testing was not performed after drawing conclusions from previously published work on the rhizoconus clade.

Methods

All the experimental procedures used for the *Conus capitaneus* venom are the same as those described for *Conus geographus* in Chapter 3. Oocyte testing and HPLC runs (both C18 and Size-exclusion) were performed in a similar manner as previously described.

Results

Purification of Conus capitaneus Venom

A 200 mg venom extraction of *Conus capitaneus* was performed in a similar manner as described for *Conus geographus* in Chapter 3. The large-scale extraction was fractionated based on hydrophobicity using a preparative C18 column, and fractions were collected in 2 minute intervals. The fractions were then screened for activity using oocytes expressing the α9α10 nAChR. This screening revealed that fraction 19 contained the active component (Fig 4.1). Five percent of the active fraction was then analyzed by HPLC using a C18 column (Fig 4.2A). Inspection of the chromatogram indicated that there were multiple overlapping peaks, suggesting that the peptides may be difficult to efficiently resolve with reversed-phase chromatography. Ten percent of the fraction was therefore also analyzed by size-exclusion chromatography (Fig 4.2B), which produced three distinct peaks based on size. Using α9α10 nAChR expressing

oocytes, these three peaks were tested and peak one was the active component (Fig 4.3), while peaks two and three did not have any activity at the concentrations tested.

Mass Spectrometry Analysis

The three peaks separated by size in the active fraction were then submitted for MALDI-TOF mass spectrometry. The first peak (active fraction) had a mass of 10966.6 Da; peak two had a mass of 3928.9 Da; and the third peak had a mass of 2803.9 Da.

Discussion

αD-conotoxins Are ~10 kDa Peptides that Non-Selectively Block Nicotinic Acetylcholine Receptors

Previous studies have identified the αD-conotoxin superfamily, a subset of conotoxins that target nAChRs. Loughnan et al. (2006) first identified the αD-conotoxins from *Conus vexillum*, and described their activity against several nAChR subtypes [18]. Subsequently Loughnan et al. (2009) [27] identified several αD-conotoxin sequences from similar *Conus* species through cDNA cloning techniques. This family of conotoxins consists of peptides that contain ten cysteine residues and are approximately 5000-5500 Da in size; the peptides form homodimers, doubling their mass to just over 10000 Da. To date, all the αD-conotoxins identified are from the rhizoconus clade of *Conus*, specifically from the species *C. vexillum*, *C. capitaneus*, *C. mustelinus*, *C. miles*, and *C. rattus* [28].

The Active Component(s) of Conus capitaneus Venom Targeted to the a9a10 Nicotinic Acetylcholine Receptor May Be an aD-Conotoxin

From this study, the venom of *Conus capitaneus* was fractionated and screened for activity on the α9α10 nAChR, resulting in an active component with a mass of 10966.6 Da;

although this is the largest intensity, there appears to be multiple other products. This mass is suggestive of the active component being an αD-conotoxin. At this point, the decision was made to not pursue this active component for several reasons. First, the activity of αD-conotoxins is potent, but fairly nonselective for nAChR subtypes. The αD-conotoxins previously characterized have shown nanomolar potency for the α7 homomer and β2* containing subtypes (* - denotes any α subunit), and weak activity for β4* containing subtypes. It should be noted that the previous published studies of αD-conotoxins were not screened for activity on α9α10 nAChRs. Although our screening of crude venom was done only on α9α10 nAChRs, Loughnan et al. reported the screening of *C. capitaneus* venom on several subtypes at 50 μg per application. They reported complete block (100%) with a slow off-rate for α7, α3β2, and α4β2 nAChRs; and "the absence of significant inhibition" for α3β4 and α4β4. The sum of information suggests that the active component from *C. capitaneus* venom against the α9α10 nAChR is most likely not going to be subtype specific. It has been reported that the αD-conotoxins are noncompetitive blockers of the ACh binding site, whereas in contrast, most α-conotoxins and αS-GVIIIB are competitive antagonists for the ACh binding site [27].

The other reason for not pursuing the active fraction is that separating the individual toxin components will be difficult due to multiple αD-conotoxins present in the fraction. Loughnan et al. (2009) report five αD-conotoxin sequences in the *C. capitaneus* venom from cDNA cloning [27]. They observed a mixture of peptides around 10,000 Da in mass present in their active fraction purified from venom [27]. This is consistent with our results from attempting to isolate the activity from the venom. As mentioned in the results, an attempt was made to separate the active fraction by size exclusion chromatography (Fig 4.2B). This was effective in creating a fraction that only contained peptides around 10000 Da in mass, but when analyzed using a C18 column (separation based on hydrophobicity), the results showed multiple peptides are still present (Fig 4.4). It is unknown how many αD-conotoxins make up the active fraction, and if

one or more is responsible for the potent activity.

The venom from *Conus capitaneus* elicited potent block of the α9α10 nAChR, and the recovery off-rate was slow. But upon investigation of the active component, the result was a large peptide, strongly assumed to be an αD-conotoxin, that presents too many challenges in isolating and identifying the active component. Additionally, from published reports for screening on other nAChR subtypes, it is highly probable that the same active component(s) contributes to the block of multiple nAChR subtypes [18] [27].

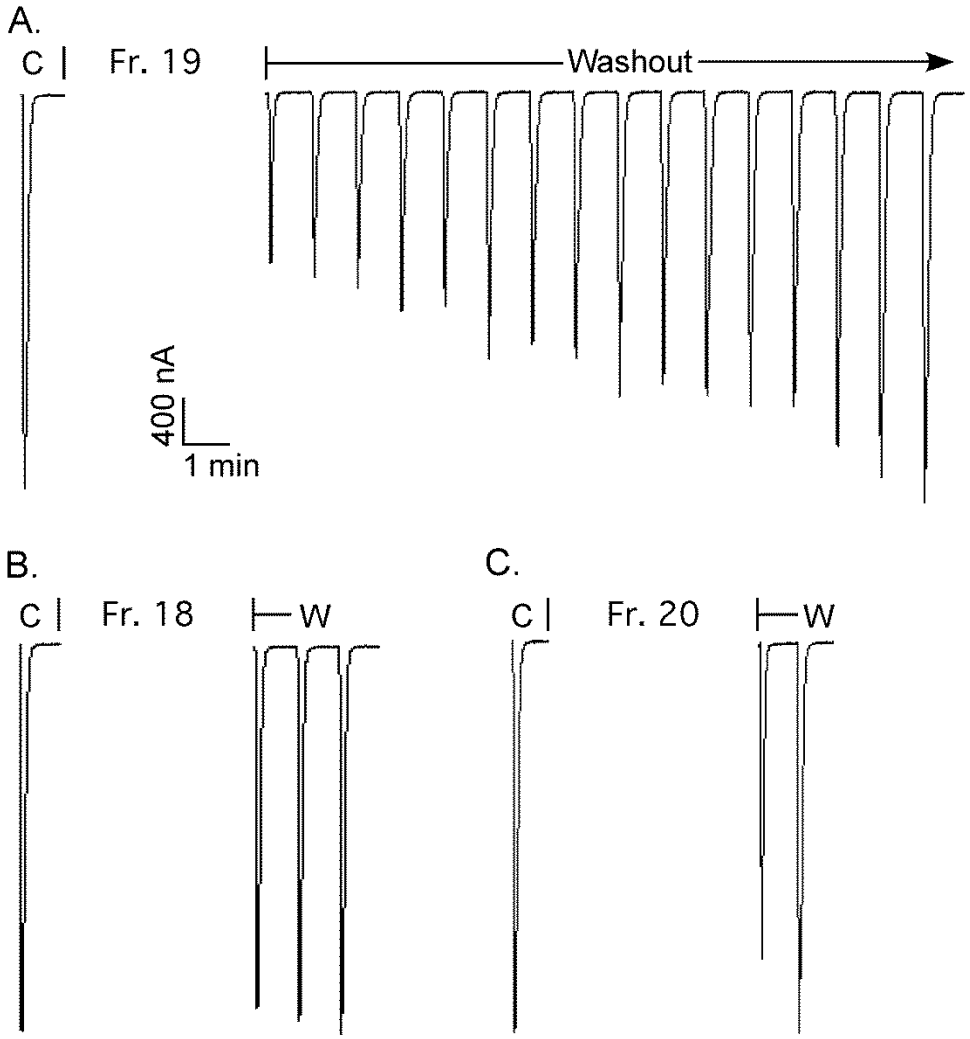

Figure 4.1 *Conus capitaneus* fractions tested for block on α9α10 nAChRs. From the 200 mg venom extraction, 10 ml fractions were collected based on hydrophobicity. For each application, a control response to 10 μM ACh was established, labeled "C". After a baseline was established, ND96 flow was paused and one two-millionth of each fraction was allowed to incubate in the well containing an oocyte expressing α9α10 nAChR for 5 min. Subsequently, ND96 flow was resumed and the fraction was washed out. **A.** Fraction 19 was the most potent; fraction 18 and fraction 20 did not exhibit block greater than 10%, **B**. and **C**.

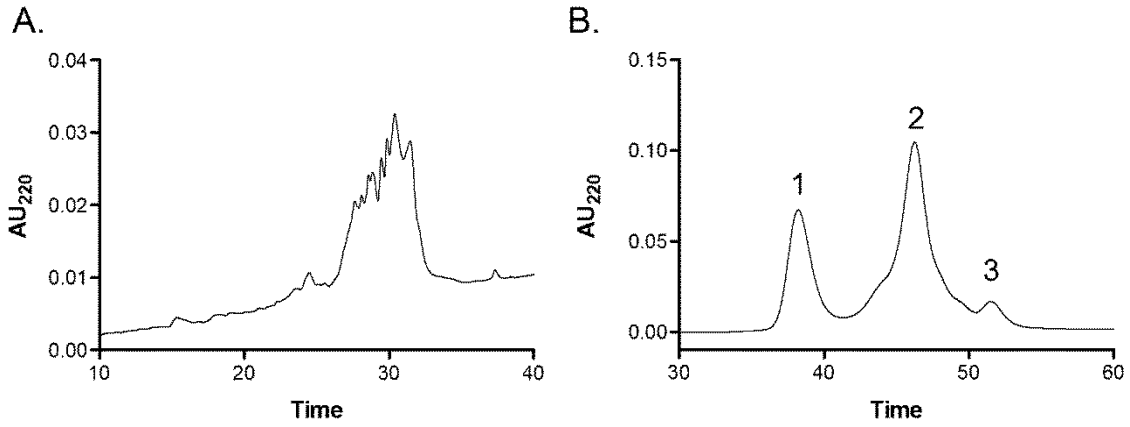

Figure 4.2 HPLC analysis of fraction 19. The active fraction was further analyzed using HPLC. A. Fraction 19 was first analyzed based on hydrophobicity using an analytical C18 column. One twentieth of the fraction was applied, and the gradient was 10% B60 (40:60:0.092 H_2O/acetonitrile/trifluoroacetic acid) to 70% B60 for 60 min (1% per min) with a flow rate of 1 ml per min. B. Size-exclusion chromatography of fraction 19. One tenth of fraction 19 was applied, and peaks were separated based on mass. An isocratic gradient was used, with B30 (70:30:0.1 H_2O/acetonitrile/trifluoroacetic acid). Peak 1 represents the active component(s).

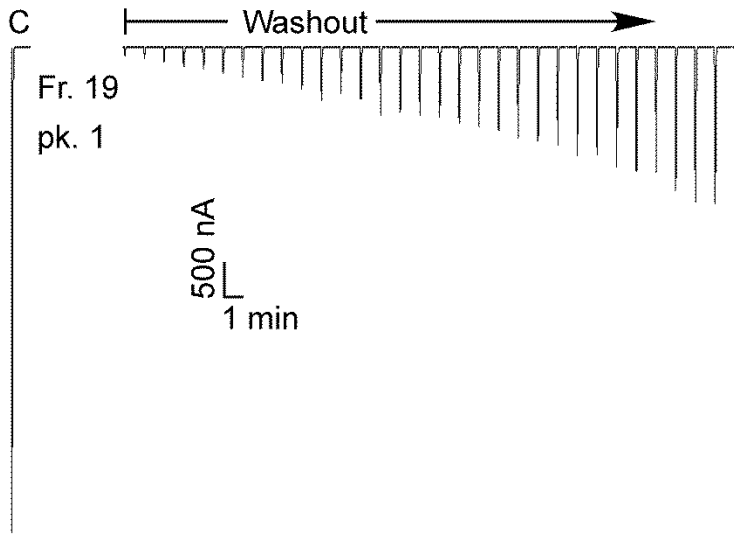

Figure 4.3 Activity of fraction 19 peak 1 following size-exclusion separation. *Xenopus laevis* oocytes expressing α9α10 nAChRS were perfused with ND96. Once per min, control applications of 1 s ACh "C" were given, until a stable baseline was established. ND96 perfusion was stopped and one twenty-thousandth of fraction 19 peak 1 was applied. After 5 min of incubation, perfusion of the oocyte with ND96 was resumed and 1 s ACh pulses were applied every min. Responses were measured following sample washout.

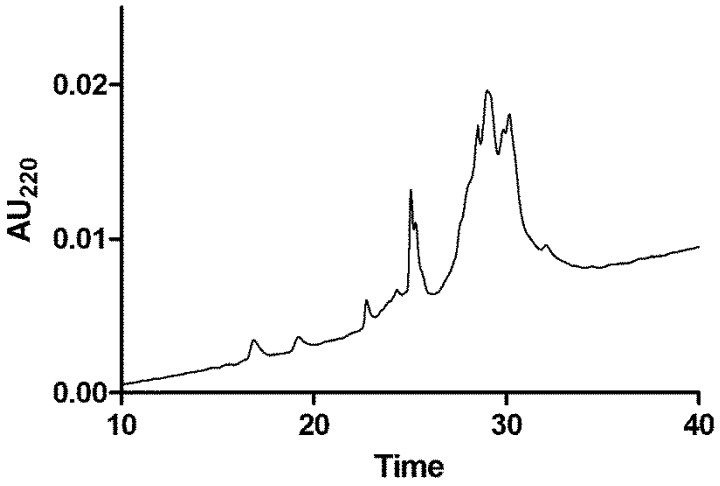

Figure 4.4 HPLC hydrophobicity analysis of fraction 19 peak 1. Peak 1 collected from size-exclusion separation exhibited potent activity when tested on α9α10 nAChRs. Peak 1 of fraction 19, with a mass of 10966.6 Da, was analyzed based on hydrophobicity a second time using a C18 column. One twentieth of the fraction was applied, and the gradient was 10% B60 (40:60:0.092 H_2O/acetonitrile/trifluoroacetic acid) to 70% B60 for 60 min (1% per min) with a flow rate of 1 ml per min. Results indicate several components are still present.

CHAPTER 5

CONCLUSION

Conclusion

Broad Array of Conotoxin Superfamilies Active on α9α10 Nicotinic Acetylcholine Receptors

Of the seven reported conotoxin superfamilies that have shown activity for nAChRs, only two superfamilies have previously been demonstrated to block the α9α10 nAChR. With this study, two more superfamilies that target nAChRs, the σ-conotoxins and the αD-conotoxins, have been identified that potently block the α9α10 nAChR.

αS-GVIIIB Is Potent for Human α9α10 Nicotinic Acetylcholine Receptors

As discussed above, the α9α10 nAChR is one of the primordial nAChRs along with the α7 and muscle subtypes. There has been some genetic drift between α9α10 nAChRs contained in the human versus the rat receptor [29]. Of the previously identified conotoxins that target α9α10 nAChRs, α-RgIA and α-Vc1.1 have both shown a significant loss in potency for the human receptor versus the rat receptor (Table 5.1) [22] [23]. As part of the extensive research with these peptides, key residues involved in the ACh binding site have been identified as critical for the loss in activity across species. As was demonstrated in Chapter 3, αS-GVIIIB is competitive with α-RgIA for the ACh binding site for the α9α10 nAChR. The activity of αS-GVIIIB was assessed for the human α9α10 nAChR, with an IC_{50} of 33.6 (26.6-42.3) nM (Fig 5.1). Thus, αS-GVIIIB is potent on both the human and rat RgIA α9α10 nAChR. In contrast, RgIA has a much lower relative potency for the human vs. rat α9α10 nAChR (Table 5.1). Since RgIA is a competitive antagonist and prevents the binding of αS-GVIIIB, the two toxins most likely bind to overlapping residues near the ACh binding domain of the rat α9α10 nAChR [30]. This suggests that one potential avenue for further study of αS-GVIIIB is to identify the key residues near the ACh binding domain that are critical, and determine if these residues are conserved across species. Having another antagonist that binds to a site that overlaps with RgIA

but with slow off-rate kinetics has the potential to further the understanding of the α9α10 nAChR.

σ–conotoxins Have Diverse Receptor Targets

With this report of the characterization and activity profile of αS-GVIIIB, there are now three known targets of σ-conotoxins. Previous work identified σ-GVIIIA, specific for the serotonin 5-HT_3 receptor and αS-RVIIIA, a peptide that preferentially blocks the muscle nAChR along with potent activity on several other subtypes [31] [17]. With αS-GVIIIB being selective for the α9α10 nAChR, the known targets of the σ-conotoxins are widening. Although the activity of these three peptides have been described, there is still much that is not known about the σ-conotoxins. All three of these peptides have been isolated from venom, with the oxidation of the disulfide bridges occurring *in vivo*. One advantage and the reason the α-conotoxins are well characterized is because they contain 4 Cys with a known disulfide pattern that can be routinely synthesized using solid phase synthesis resulting in peptides that are similar to α-conotoxins purified from the venom. The σ-conotoxin superfamily is defined by the sequence spacing of 10 Cys residues, but the disulfide pattern is not known. Identifying the disulfide framework is a logical next step in advancing the understanding of the σ-conotoxins. This can facilitate the synthesis of σ-conotoxins *in vitro*, utilizing solid phase peptide synthesis. Successfully making a synthetic peptide that is similar in activity to one of the venom isolated compounds would be instrumental in establishing protocols that could be used for the several σ-conotoxin sequences that have been identified from genetic data.

New Activity Described for αD-conotoxins

The αD-conotoxins have previously been reported as noncompetitive blockers of several nAChR subtypes [18]. This study suggests that the αD-conotoxins are potent for α9α10 nAChRs

in addition to the many other nAChRs they target. Although not definitively shown, there is strong suggestive evidence, based on mass and hydrophobicity profile, that the active component(s) of the venom is an αD-conotoxin. The rhizoconus clade is unique in that there have not been any reported α-conotoxins from the six species that are part of this group. With the absence of α-conotoxins, the major class of conotoxins that target nAChRs in some species, the rhizoconus clade has utilized the αD-conotoxins to target the nAChRs in their prey [28]. This illustrates the importance that *Conus* has placed on having components in the venom that potently block nAChRs.

Table 5.1 IC_{50} of rat versus human α9α10 nAChRs

	Rat α9α10	Human α9α10	Ratio	Reference
α-RgIA	1.49 nM	494 nM	331.5	[22]
α-Vc1.1	70.0 nM	975.4 nM	13.9	[32]
αS-GVIIIB	9.79 nM	33.6 nM	3.4	This Study

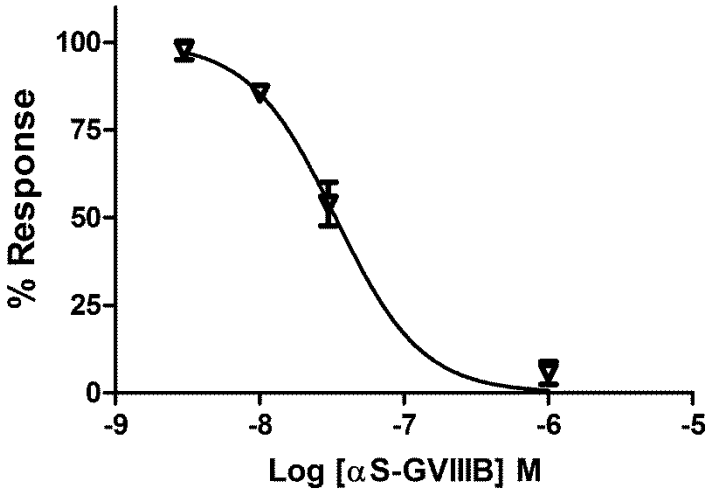

Figure 5.1 αS-GVIIIB concentration-response curve for human α9α10 nAChRs. αS-GVIIIB was tested on *Xenopus laevis* oocytes microinjected with an equal ratio of human α9 and human α10 nAChR subunits. αS-GVIIIB blocked the human α9α10 nAChR with an IC_{50} of 33.6 (26.6-42.3) nM and a Hill slope of 1.5 (0.9-2.0). For the 3 nM, 10 nM, and 30 nM data points, a solution containing the toxin was perfused with 1 s ACh applications occurring once per min, until an equilibrium of block was achieved. For the 1 μM data point, ND96 flow was paused for 5 min and 1μM αS-GVIIIB was allowed to incubate in the oocyte well. ND96 flow was resumed and 1 s ACh pulses were applied per min.

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